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**Establishment of a Gene Linkage Map in Lentil and
Chickpea Using Isozyme and DNA Markers**

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Establishment of a Gene Linkage Map in Lentil and Chickpea
Using Isozyme and DNA Markers

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C. ABSTRACT

Polymorphic molecular markers, including both allozymes and DNA restriction fragments, were identified in the parents of two interspecific crosses: *Lens culinaris* x *L. ervoides* and *Cicer arietinum* x *C. reticulatum*. A total of 62 such markers were found in the first cross and 30 in the latter. Segregation analysis of the markers in F₂ and F₃ populations confirmed the simple genetic basis for each polymorphism. Linkage relationships among the markers were established, and the resulting chromosomal maps were compared with each other and with that previously established for the garden pea (*Pisum sativum*). Large portions of the lentil map appeared to display synteny with that of the garden pea. Little synteny could be observed between the linkage map of chickpea and that of either of the other crops; however, the *Cicer* map contained far fewer markers, possibly preventing the recognition of parallel linkage groups. The similarities between the garden pea and lentil maps implies that despite the presence of a number of translocations and inversions within each of the genera, much of the genome has remained intact within the Viceae.

One RFLP observed between the two *Cicer* parents was determined to be a portion of the chloroplast DNA. This polymorphism segregated in the F₂ even though the original cross was always made with the same maternal parent (*C. arietinum*). It was concluded that cpDNA can show biparental inheritance in this interspecific cross. Another surprising result in *Cicer* was the discovery that two plastid-specific aldolase isozymes were present in extracts from leaf tissue. We demonstrated that these were coded by distinct genes and believe that a portion of the *Cicer* genome has been duplicated.

In order to facilitate the development of a linkage map for lentil, attempts were made to isolate tertiary trisomics in the species and match them to linkage groups by *in situ* hybridization. Trisomics were isolated from four different crosses, but only those from one cross generated trisomic plants that were vigorous and fertile. *In situ* hybridization using the pea ribosomal DNA probe demonstrated that the position of the ribosomal genes could be observed using non-radioactive assays but that the rDNA cluster was not on the tertiary trisomic. We were not able to visualize the position on single copy genes using this technique.

D. OBJECTIVES OF ORIGINAL RESEARCH PROPOSAL

Five objectives were stated in the original proposal:

1. Assess genetic diversity at isozyme loci and DNA restriction fragments in *Lens* and *Cicer* germplasm.
2. Construct a linkage map in *Lens* primarily consisting of molecular markers.
3. Develop a set of trisomic tester lines in lentil.
4. Initiate development of a linkage map in chickpea.
5. Compare the linkage map of garden pea with those developed for lentil and chickpea to determine if linkage groups conserved among the three taxa could be identified.

A number of factors, most specifically a significant reduction in the length and amount of support originally requested, forced a revision of these objectives even before the project was initiated. We did not perform an extensive survey of the genetic diversity in either genus. Instead we identified two crosses that we felt would include the greatest level of genetic diversity and still produce reasonably fertile progeny. Linkage relationships among isozyme loci in lentil had been partially determined before the project was started, permitting us to focus on the further development of the lentil linkage map relatively early in the project.

Development of general linkage maps for *Lens* and *Cicer* from interspecific crosses and comparison of these with the linkage map for *Pisum*

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INTRODUCTION

Chromosomal linkage maps have long served as a convenient method for summarizing much of the genetic information known about a species. Previously, the development of even a partial linkage map for a vascular plant required considerable time, particularly in the generation and scoring of appropriate segregating populations. Thus, until recently, only a few of the most intensively studied species such as tomato (*Lycopersicon esculentum*), maize (*Zea mays*), and garden pea (*Pisum sativum*) had relatively complete linkage maps. However, with the discovery of allozyme and, more recently, DNA restriction fragment length polymorphisms, the number of segregating markers that can be conveniently scored in a progeny has increased tremendously, permitting detailed linkage maps to be established for a considerable number of plant species (Bernatzky and Tanksley, 1986; Helentjaris et al., 1986; Landry et al., 1987; McCouch et al., 1988; Chang et al., 1988; Gebhardt et al., 1989). Despite this great increase in efficiency, the

generation of a linkage map *de novo* still requires considerable effort and expense, and it continues to be appropriate to look for additional approaches that will streamline the process.

The Viceae is a small tribe of north temperate legumes with four significant crops: garden pea (*Pisum sativum*), lentil (*Lens culinaris*), faba bean (*Vicia faba*), and sweet-pea (*Lathyrus sativus*). Chickpea (*Cicer arietinum*) is a fifth crop closely allied with this tribe, although currently placed in its own tribe, Cicereae. Of these crops, only the garden pea has been investigated sufficiently to possess a chromosome linkage map covering all seven chromosomes (Blixt, 1974). The strong chromosomal homology that has been observed among wheat and its relatives (Hart, 1979) and between tomato and potato (Bonierbale et al., 1988) indicates that some of the linkage groups identified in *Pisum* also may occur in other Viceae and perhaps even in *Cicer*. However, in order to document syntenic regions in different genomes it is necessary to have available homologous markers mapped on both. Fortunately, homologies among loci coding specific isozymes are relatively easy to establish (Weeden and Wendel, 1990) as are those among certain other classes of genes that can be scored using RFLP technology. A large number of such markers already have been mapped in the garden pea (Weeden and Wolko, 1990). If these same markers could be mapped in lentil or some other closely related legume, a good estimate of the syteny present within the two genomes could be made. In this paper we present map positions for 52 markers in lentil and 18 in chickpea. By combining these data with results from other laboratories, we should be able to determine if many linkage groups are shared among these genera.

Materials and Methods

Plant material: The two lines used for the interspecific cross in *Lens* were *L. culinaris* (Ladizinsky #7) and *L. ervoides* (Ladizinsky #32). The two lines are known to differ by a reciprocal translocation but otherwise generate relatively fertile F₂ progeny (Cohen et al., 1984). Dr. D. Zamir generously donated seed from approximately 80 individual F₂ plants, the F₂ already having been scored for 18 morphological and isozyme marker loci (Tadmor et al., 1987). Ten seeds from each F₂ plant were germinated and 63 of these F₂ progenies gave a sufficient number of F₃ plants for our analyses.

Morphological and allozyme phenotypes were determined on five F₃ plants. The morphological markers yellow cotyledon (Yc), epicotyl color (Gs), pod indehiscence (Pi), seed coat spotting (Scp), and seed ground color (Tgc) were scored as described by Havey and Muehlbauer (1989). DNA was extracted from pooled leaf tissue of all F₃ plants from a particular F₂. In this way the genotype of the F₂ could be determined both for isozyme loci and RFLPs by extrapolation from the F₃ data.

The two lines used for the interspecific cross in *Cicer* were a kabuli type *C. arietinum* (PI360177) and a *C. reticulatum* line derived from PI489777.

Isozyme phenotypes were determined on individual F₂ plants and confirmed on 2-4 F₃ plants derived from each F₂. The morphological polymorphisms in flower color (P) and pod indehiscence (Pi) as well as the isozyme phenotypes are described in Kazan et al. (in prep.). DNA was isolated from pooled leaf tissue from 5-10 F₃ plants. A total of 50 F₂ plants were used in our mapping study.

Isozyme analysis: The generation of extracts from leaf, seed, or root tissue in both progenies was accomplished as described for pea in Weeden and Marx (1984, 1987). Four gel systems were used for the resolution of the various

enzyme systems. System I was the tris-citrate/lithium borate pH 8.1 buffer of Selander et al (1970). System II was the trizma-histidine system of Weeden (1987). System III was the histidine-citrate buffer of Cardy et al. (1980), and System IV was an N-amino-morpholine buffer described by Clayton and Tretiak (1972).

For *Lens*, the leucine aminopeptidase (LAP), aspartate aminotransferase (AAT), peroxidase (PRX) and rubisco isozymes were resolved in System I; the alkaline B-galactosidase (GAL8), acid phosphatase (ACP), and alanine aminotransferase (ALT) in System II; and the NADH-diaphorase (DIA), phosphoglucomutase (PGM), 6-phosphogluconate dehydrogenase (6PGD), and shikimate dehydrogenase (SKDH) on System III. For *Cicer*, the aconitase (ACO), alanine aminopeptidase (AAP), glucosephosphate isomerase (GPI), PGM, seed protein, and ACP were resolved on System I; the ALT, DIA and adenylate kinase (ADK) on System III, and the 6PGD, fluorescent esterase (EST) and acidic B-galactosidase (GAL4) on System IV.

RFLP analysis: High molecular weight DNA was obtained from young leaves of six- to eight-week old seedlings using a cTAB extraction essentially as described by Doyle and Doyle (1987). Ten micrograms of DNA was used for restriction digests. DNA from the parents was screened for RFLPs using the enzymes EcoRI, EcoRV, BamHI, BglII, and HindIII. Restriction digests were performed for 6-8 hours at 37 C, in buffers supplied with the enzymes. The digests were stopped by adding one tenth volume loading buffer (1mM EDTA pH 7.5, 5% glycerol, and 0.1% bromphenol blue). Electrophoresis of digested DNA was carried out in 0.9% agarose gels until the bromphenol blue front had migrated 15 cm. Gels were stained in ethidium bromide (0.5 ug/ml) and photographed over UV light (302 nm). The DNA was denatured by soaking in

0.4 N NaOH with 0.6 M NaCl for 20 minutes and transferred to Genescreen filters using the alkaline blot procedure of Reed and Mann (1985).

Random genomic and cDNA clones from lentil (cv. Brewer) were obtained as described in Havey and Muehlbauer (1989). In addition, a number of cDNA clones from garden pea were used as well as three clones from other sources (Table 1). Except for pHA2, which was labelled in entirety, the plasmids were first cut with enzymes that would remove the insert, and the insert was separated from the vector on low melting point agarose gels before oligolabeling with ^{32}P by the method of Feinberg and Vogelstein (1983). Hybridization was performed overnight at 65 C in 0.5 M NaHPO_4 , pH 7.2, containing 10X Denhardt's solution, 1 mM EDTA, 0.1 mg/ml sonicated calf thymus DNA, 0.6% (w/v) SDS, and 2.5% (w/v) dextran sulfate. Filters were washed three times with 2x SSC containing 0.1% SDS at 65 C for 30 minutes. Filters were placed on X-ray film (Kodak XAR-5) with DuPont Cronex Lightning Plus intensifying screens at -70 C for 2-4 days.

Linkage analysis: Linkage between morphological, isozyme, and RFLP markers was estimated from F_2 segregation data using the method of maximum likelihood as applied by the LINKAGE-1 program (Suiter et al., 1983). Linkage groups were constructed from estimates of recombination between pairs of markers. For comparisons in which data were available for less than 50 F_2 individuals, linkages greater than 20 map units or with a P value of greater than 0.01 were not reported. For RFLP data in particular, we would often perform a preliminary analysis for linkage with other markers after obtaining data on 20-40 F_2 plants. If the RFLP showed close linkage to one of the previously scored markers or mapped between two markers already known to be linked we did not attempt to complete the data set for the marker.

RESULTS

In the *L. culinaris* x *L. ervoides* progeny 65 segregating markers were scored (Table 2). These included 46 new markers (Table 2) not reported by Tadmor et al. (1987). Approximately 20% of the markers gave segregation ratios deviating significantly from expected Mendelian ratios of 3:1 or 1:2:1. However, half of these markers were coded by loci on the chromosomes involved in the reciprocal translocation. The normal segregation ratios observed for most of the other markers suggests that the majority of the chromosomes pair and assort randomly. We therefore expected that most of the linkages we identified would represent true association of markers on single chromosomes.

Joint segregation analysis of the polymorphisms revealed fifteen multilocus linkage groups (Figure 1) at a probability level of $p \leq 0.01$. Two of these linkage groups (#1 and #8) did not resolve into linear maps. Linkage group #1 is known to contain the break point (BP) for the reciprocal translocation. Markers on this linkage group may thus be expected to display distorted segregation ratios. The sequence of loci shown for group #1 in Figure 1 is what we believe to be the most likely order of the loci. Two markers, PMH95 and Dia-2, showed linkage to Rrn but could not be satisfactorily placed on the map relative to the flanking loci. In linkage group #8, the Act-2 locus gave recombination frequencies with CMH34 and Skdh that indicated that it would map near Est-1. However, Act-1 and Est-1 assorted independently. We felt that more data are necessary before Act-2 can be accurately located on this linkage group. All other linkage groups gave relatively consistent map distances among the markers.

In the *Cicer* cross, 30 segregating polymorphisms were observed (Table 3). Several polymorphisms gave segregation ratios deviating significantly from expected ratios. Certain of these (SP-1, ALDO-1, ACO-2, and ADK) gave easily scored phenotypes. Segregation distortion at the respective loci was therefore presumed to reflect abnormalities in pairing or selective loss of certain chromosomal segments. Other phenotypes (GAL-2, DIA, ALT) were occasionally difficult to resolve, and for some plants could not be determined despite repetitive analyses. In this latter group, at least some of the skewing could have been caused by the inaccurate scoring of the phenotypes. Joint segregation analysis identified seven multilocus linkage groups (Figure 2). These included 14 loci encoding proteins identified by starch gel electrophoresis and three RFLPs.

DISCUSSION

The lentil linkage map was significantly expanded by examining simple genetic polymorphisms in the cross *Lens culinaris* x *L. ervoides*. The linkage data generated for F₂ and F₃ populations from this cross confirmed much of the genetic map previously established from studies on *L. culinaris* intraspecific crosses or *L. culinaris* x *L. orientalis* progenies (Havey and Muehlbauer, 1989; Vaillancourt, 1989; Muehlbauer et al., 1989). The *L. culinaris* x *L. ervoides* progeny had far more polymorphisms segregating (62) than any of the previous crosses used to develop the linkage map, and it should be possible to map additional RFLPs relatively quickly using the markers already available in combination with recombinant inbred lines generated from this cross.

A number of morphological and isozyme loci that were not segregating in the *L. culinaris* x *L. ervoides* progeny or did not show sufficiently close linkage to meet our criteria have been mapped by others (Tadmor et al., 1987; Havey and Muehlbauer, 1989; Vaillancourt, 1989). By including many of these previous results, a generalized linkage map for *Lens culinaris* can be developed (Figure 3). In this map our linkage group #1 has been separated by division at the break point into two linkage groups referred to as group I and II. The Sp-2---CMH52c segment (our linkage group #14) can be added to group I on the basis of the data of Tadmor et al (1987). On the same basis our linkage group #2 can be added to group II. Havey and Muehlbauer (1989) reported linkage between EMH1 and markers on linkage group III. We observed weak linkage between the EMH1---PMH110b segment and Aps-1 but not to either of the flanking markers, CMH41 or Pi.

Several other inconsistencies with previous results were noted. Tadmor et al. (1987) reported that the chromosome containing the nucleolar organizer region (and presumably the ribosomal DNA gene cluster) was not one of those involved in the translocation between *L. culinaris* #7 and *L. ervoides* #32. However, we found that the ribosomal DNA cluster mapped very near the break point, implying that the satellite chromosome was involved in the translocation. An alternative explanation would be that the ribosomal DNA cluster or some locus tightly linked to it interacted with a gene or genes in the region of the translocation in such a way that recombinant phenotypes were strongly selected against. At present we have no reason to favor the latter alternative. We also had to alter the order of markers on this linkage group slightly to conform to our results. However, the gene order must be considered tentative until a more thorough study can be performed with parents lacking chromosomal rearrangements. One linkage reported by Havey and

Muehlbauer (1989), that between EMH14 and CMH81, was not verified in our study. We found EMH14 to be linked to Scp and CMH65a, whereas CMH81 displayed linkage to Sp-4. This anomaly may indicate another chromosomal rearrangement between the material used by Havey and Muehlbauer and the lines used in the present study.

When this lentil linkage map is compared with the map recently published for the garden pea (Weeden and Wolko, 1990) a number of conserved linkages (indicated by boxes in Figure 3) as well as several rearrangements are apparent. The region from the leghemoglobin cluster to CMH52c on linkage group I is similar to the sequence of genes from D to CMH52b on chromosome I of pea, although several inversions must be postulated. The presence of the ribosomal gene cluster on lentil linkage group I does not correspond to either of the clusters in pea, those in pea being on chromosome IV (linked to PMH119 and CMH41) and on chromosome VII (linked to Pgd-p and CMH58).

The Aps---Pi---Gal-1 set on lentil group III is similar to a Gal-1---Acp-3---Dpo set on pea chromosome III. The Pi and Dpo genes both control pod dehiscence, and it would be particularly interesting if these should prove to be homologous. The Gal-1 genes probably are homologous because both code for the single beta-galactosidase enzyme with an alkaline pH optimum in each taxon. The homology between the two acid phosphatase loci remains to be demonstrated.

Three other comparisons are of much more certain syntenic. The region covered by CMH69 to Lap-1 on linkage group IV has a counterpart on chromosome II of pea. The entire linkage group VII may be represented on pea chromosome VII if Lap-2 of lentil is homologous with Pep-3 of pea. Finally, the sequence Gal-2 though the Cab cluster (linkage group VIII) is

paralleled in pea on chromosome VII. Thus in pea (where the chromosomal linkage groups are better defined) major portions of at least 4 of the 14 chromosome arms show significant synteny with linkage groups of lentil.

The chickpea map is still rudimentary, although considerable genetic variability between the two parental lines still remains to be used for mapping. It is possible that the entire linkage map could be generated by continued work on the *Cicer arietinum* x *C. reticulatum* cross. At least as developed from our data, the chickpea map does not contain any obvious regions of synteny with either the pea or lentil map. Adenylate kinase has not displayed polymorphism in either pea or lentil, and hence the corresponding locus has not been mapped. The homology of the chickpea aconitase and beta-galactosidase loci with those in lentil and pea is uncertain, although Gal-1 of chickpea encodes an enzyme with an acid pH optimum and thus the locus is not homologous with Gal-1 in either pea or lentil. Neither the Pgm-p locus in lentil nor that in pea is linked to an esterase locus. There is a linkage between a diaphorase and a beta-galactosidase locus on chromosome II in pea, but the homology with Dia and Gal-3 in chickpea has not been confirmed.

The most likely candidate for a shared linkage group is the Skdh---Est-1---Aldo---Gal-2 group in pea with a similar, perhaps duplicated set in chickpea. It is known that the plastid-specific aldolase in chickpea is coded by duplicate genes (Kazan et al., submitted) and it appears that Skdh and an esterase locus also may be duplicated. These findings may indicate that a segment of a chromosome containing all three loci has been duplicated in chickpea. More direct evidence for the presence of this linkage group in chickpea was obtained by P. Gaur (unpublished Ph.D. thesis) who described a linkage group including an aldolase, a shikimate dehydrogenase, an esterase, a phosphoglucomutase, a 6-phosphogluconate dehydrogenase and a peptidase

locus in a *C. arietinum* x *C. reticulatum* cross. This linkage group matches that for pea chromosome VII, and lentil groups VII and VIII.

We are optimistic that other examples of synteny among the chickpea, lentil and pea genomes will be revealed as more homologous markers are identified. Our approach has been to maximize the genetic diversity in the original parental lines and generate linkage maps from only one or a few crosses. In both lentil and chickpea the relatively low level of intraspecific polymorphism for molecular markers has forced us to use interspecific crosses. The disadvantage of using such crosses is the possibility of reporting pseudolinkages produced by structural rearrangements in the chromosomes. Fortunately the chromosomal repatterning between *Lens culinaris* and *L. ervoides* was known before we initiated the mapping study. Even the position of the translocation breakpoint on the linkage map had been determined. The segregation patterns of additional markers in this cross revealed that most of the remainder of the genome segregated normally, suggesting that most of the linkages observed in the analysis represent intrachromosomal linkages rather than pseudolinkages caused by chromosomal rearrangements. This conclusion is strongly supported by the similarity between our map and those reported by Havey and Muehlbauer (1989), Vaillancourt (1989) and Muehlbauer et al. (1989). These latter maps were based on data from crosses among the more closely related species *L. culinaris*, *L. orientalis*, and *L. odemensis*.

The current lentil linkage map, which spans approximately 550 cM, probably includes markers on most, if not all, of the 14 chromosomal arms. Of the 62 markers listed in Table 2 only 6 (GS299, Dia-1, CMH52b, EMH8, Act-3, and Tgc) could not be assigned to a multilocus linkage group. If we assume that we would have observed linkage if any of these markers were within 15 cM of

another marker there must be at least 180 map units represented by these single loci. The total map distance covered by both the multilocus linkage groups and the single loci, about 750 cM, is similar to the 850 cM map presently described for *Pisum sativum*.

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CAPTIONS FOR FIGURES

Figure 1. Linkage groups identified in *Lens* using the interspecific cross *L. culinaris* x *L. ervoides*.

Figure 2. The seven linkage groups identified in *Cicer* using the interspecific cross *C. arietinum* x *C. reticulatum*.

Figure 3. Current linkage map for lentil, including data from both intraspecific and interspecific crosses. Boxed regions indicate regions of synteny with the linkage map for the garden pea.

Table 1. Source and characterization of probes derived from
species other than *Lens culinaris*.

<u>Plasmid</u>	<u>Nature of insert</u>	<u>Reference</u>
pHA2	pea ribosomal DNA repeat	Jorgensen et al. (1987)
pAB96	pea chlorophyll a/b binding protein gene	Broglie et al. (1981)
GS185	pea plastid-specific glutamine synthase	Tingey et al. (1987)
GS299	pea cytosolic glutamine synthase	"
GS341	pea nodule-specific glutamine synthase	"
rp122	pea chloroplast ribosomal protein-22	J. D. Palmer
Nco1	pea plastid-specific glyceraldehyde 3-P dehydrogenase	R. Cerff et al. (1986)
pALB	alfalfa leghemoglobin	K. Dunn et al. (1988)
pSAc3	soybean actin	R. Meagher
pCIB	squash nitrate reductase	Crawford et al. (1986)

Table 2. Segregation of single gene markers in the F₂ from the cross *Lens culinaris* x *L. ervoides*.

Locus	N	No. individuals with designated phenotype ¹			Ratio	χ^2
		Lc	H	Le		
Aat-m	61	17	32	12	1:2:1	0.97
Aat-mb	42	11	19	12	1:2:1	0.43
Aat-p	62	6	32	24	1:2:1	10.51**
Acon	63	11	33	19	1:2:1	2.17
Act-1	40	6	27	7	1:2:1	4.95
Act-2	31	22		9	3:1	0.27
Act-4	32	27		5	3:1	1.50
Aps-1	59	15	29	15	1:2:1	0.05
Cab	26	3	20	3	1:2:1	7.54*
CMH3	11	2	7	2	1:2:1	0.82
CMH33	27	8	11	8	1:2:1	0.93
CMH34	31	4	22	5	1:2:1	5.52
CMH41	24	6	12	6	1:2:1	0.00
CMH52a	24	5	10	9	1:2:1	2.00
CMH52b	23	4	14	5	1:2:1	1.17
CMH52d	34	12	15	7	1:2:1	1.94
CMH58	32	7	17	8	1:2:1	0.52
CMH65a	61	14	--	47	1:3	0.14
CMH65b	50	45	--	5	3:1	6.00*
CMH71	38	5	24	9	1:2:1	3.45
CMH81	44	19	21	4	1:2:1	10.32**
Dia-1	42	11	21	10	1:2:1	0.05
Dia-2	43	8	22	13	1:2:1	1.17
EMH1	29	6	11	12	1:2:1	4.17
EMH5	48	13	29	6	1:2:1	4.12
EMH8	35	9	19	7	1:2:1	0.48
EMH14	31	10	17	4	1:2:1	2.61
EMH25	20	2	15	3	1:2:1	5.10
Est	59	14	25	20	1:2:1	2.59
Ga11	40	10	24	6	1:2:1	2.40
Gdh	35	5	14	16	1:2:1	8.31**

Locus	N	Lc	H	Le	Ratio	χ^2
Gs	62	6	30	26	1:2:1	12.96**
GS185	19	10	8	1	1:2:1	9.09**
GS299	33	20	--	13	3:1	3.74
GS341	27	7	15	5	1:2:1	0.63
Lap-1	62	19	37	6	1:2:1	7.77*
Lap-2	54	9	26	19	1:2:1	3.78
Lb	28	4	20	4	1:2:1	5.14
Ncol	37	11	15	11	1:2:1	1.32
Pep-1	62	16	25	21	1:2:1	3.13
Pgd-p	63	13	32	18	1:2:1	0.80
Pgm-p	60	7	39	14	1:2:1	7.03*
Pi	52	6	--	46	1:3	0.02
PMH68	41	10	24	7	1:2:1	1.63
PMH95	21	6	11	4	1:2:1	1.46
PMH110a	20	5	9	6	1:2:1	0.30
PMH110b	22	5	10	7	1:2:1	0.55
PMH110c	20	5	--	15	1:3	0.00
PMH111b	37	13	16	8	1:2:1	2.05
PMH111x	48	38	--	10	3:1	0.44
PMH111y	48	40	--	8	3:1	1.78
Prx1	62	21	31	10	1:2:1	3.90
Prx3	62	14	39	9	1:2:1	4.93
RbcS	52	11	31	10	1:2:1	1.96
Rpl22	47	13	23	11	1:2:1	0.19
Rrn	55	7	37	11	1:2:1	7.14*
Scp	62	15	--	47	1:3	0.21
Skdh	58	10	34	14	1:2:1	2.27
Sp-2	62	14	30	18	1:2:1	0.58
Sp-4	63	20	31	12	1:2:1	2.04
Tgc	63	52		11	3:1	1.91
Yc	58	5	--	53	1:3	8.29**

¹Phenotypic designations: Lc = *Lens culinaris* parent, H = F₁ heterozygote, Le = *Lens ervoides* parent.

Table 3. Segregation of polymorphism in the cross *Cicer arietinum* x *C. reticulatum*.

Locus	N	No. with designated phenotype ¹			Expected ratio	χ^2
		Ca	H	Cr		
Aap-1	34	12	17	5	1:2:1	2.88
Aco-1	50	13	20	17	1:2:1	2.64
Aco-2	49	7	23	19	1:2:1	6.06*
Acp-2	48	8	25	15	1:2:1	2.12
Acp-3	43	14	18	11	1:2:1	1.56
Acp-4	46	5	27	14	1:2:1	4.91
Adk	47	5	22	20	1:2:1	9.77**
Aldo-p1	49	7	18	24	1:2:1	15.24**
Alt	27	5	20	2	1:2:1	7.85*
CMH34	13	2	8	3	1:2:1	0.85
CMH52	32	7	16	9	1:2:1	0.25
CMH58	15	3	7	5	1:2:1	0.60
CMH69	11	1	6	4	1:2:1	1.72
Dia	35	8	11	16	1:2:1	8.49*
Est-c	40	8	20	12	1:2:1	0.80
Gal-1	40	6	24	10	1:2:1	2.40
Gal-2	43	4	22	17	1:2:1	7.88*
Gal-3	35	7	16	12	1:2:1	1.68
Gpi-c	50	16	22	12	1:2:1	1.36
GS299a	12	7		5	3:1	1.78
GS299b	12	2	7	3	1:2:1	0.50
GS299c	23	8	10	5	1:2:1	1.17
GS299d	10	9		1	3:1	1.20
Nr	18	4	10	4	1:2:1	0.22
P	50	13	--	37	1:3	0.03
Pgd-c	48	8	29	11	1:2:1	2.45
Pgm-p	50	10	26	14	1:2:1	0.72
Pi	45	10	--	35	1:3	0.18
rpl-22	14	5	7	2	1:2:1	1.29
Sp-1	44	4	21	19	1:2:1	10.32**

¹Phenotypic designations: Ca = *Cicer arietinum* parent, H = F₁ heterozygote,Cr = *Cicer reticulatum* parent.

Tertiary trisomics in lentil

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Introduction

Aneuploid lines are valuable tool for assigning individual genes or linkage groups to chromosomes (Khush 1973). In diploid organisms individuals with $2n-1$ chromosome number are usually not viable and the main aneuploids which can be obtained are trisomics. So far, no trisomic plants have been reported in lentil. Our attempt to produce primary trisomics by crossing a diploid line with its colchicin induced autotetraploid derivative and back crossing the $3x$ plant with pollen of the diploid line have been unsuccessful because the $3x$ embryos died about a week after fertilization. This embryo breakdown occurred in more than six hundred crosses in both cross directions. Spontaneous triploid sectors however may be detected on diploid plants but have not set any seed upon back-crossing with normal pollen (Malaviya and Shukla 1983). Tertiary trisomics may occur spontaneously among progeny of translocation heterozygotes. Chromosome rearrangements are rare in the cultivated lentil but relatively common in wild

lentils. We report here the occurrence and behavior of tertiary trisomics that have been detected among progeny of hybrids involving the cultivated lentil Lens culinaris and various wild forms.

Materials and methods

Trisomic plants were detected among F₂ individuals of the following crosses: L. culinaris (no. 13) X L. orientalis (no. 24), L. culinaris (no. 2) X L. orientalis (no. 42), L. culinaris (no. 2) X L. orientalis (no. 133), L. culinaris (no. 2) X L. ervoides (no. 32). For karyotype study seeds were germinated in petri dishes at 15° C. Primary roots of about 1 cm were placed in cold water (4° C) for 24 h, fixed in 3:1 absolute ethanol - acetic acid for 12 h, hydrolized in 1N HCl for 10 min at 60° C and stained with Feulgen. For meiotic study buds at appropriate stage were fixed in 3:1 and stained with acetocarmine. Pollen fertility was determined by staining mature anthers with acetocarmine. Regularly shaped and evenly stained grains were considered viable.

Results

The lentil karyotype contains three pairs of sub-metacentric chromosomes, three pairs of acrocentric chromosomes and one pair of metacentric chromosome with a secondary constriction, very close to the centromer. In a search for tertiary trisomics in lentil it is possible not only to detect

plants with $2n=15$ but also to characterize the morphology of the extra chromosome.

Trisomics of *L.culinaris*, no.13 X *L.orientalis*, no.24 cross

This particular hybrid was heterozygous for a reciprocal translocation which involved a submetacentric and acrocentric chromosomes. At metaphase I a heteromorphic quadrivalent was observed in many cells. In a previous study (Baruchin and Ladizinsky 1983) it has been established that the long arm of the acrocentric chromosome was involved in the rearrangement. Among 60 progeny of this hybrid one had a $2n=15$ chromosome number and the extra chromosome was submetacentric. This plant had only two branches and produced 15 seeds and some of the pods had a slit in their ventral side (Fig. 1). Only limited number of M1 cells were examined of which two had 5 bivalents and a pentavalent (5II+V) Fig. 2, indicating that it was a tertiary trisomic, namely that the extra chromosome possessed the native arm of the submetacentric chromosome and a translocated segment of the acrocentric chromosome. Three of the 15 seeds collected on that plant gave rise to trisomic plants. Table 1 shows the number of seeds collected from each of these $2n=15$ plants and the transmission rate of the extra chromosome. In all the 44 derived trisomic plants the extra chromosome was submetacentric. Chromosome association at M1 was study in 17 trisomic plants (Table 2). All were identified as tertiary trisomics according to cells with 5 bivalents and a pentavalent or occurrence of heteromorphic trivalents that were composed of two acrocentric and

one submetacentric chromosomes (Fig. 2). Of the 112 homomorphic trivalents observed 95 were V shaped and only 17 formed straight chain in which only the two terminal centromeres were active. Twenty one heteromorphic trivalents were composed of two acrocentric and one submeracentric chromosomes (Fig. 2). These trivalents were Y shaped or of the fry pan type and only one formed straight chain. In cells with 7II+I configuration the univalent was always submetacentric. Assuming that the translocated chromosome was left unpaired the proportions of the pentavalent and the various trivalent configurations indicate chiasma frequency in the various regions of the extra chromosome. In the pentavalents both the native and the translocated arms were involved in chiasma but in the homomorphic trivalents only the native arm was involved, while the translocated arm was associated in chiasma in the heteromorphic trivalents. Accordingly, chiasma frequency was 0.79 and 0.63 in the native and the translocated arms, respectively. In the F_1 hybrid chiasma frequency in these chromosome segments was 0.95 and 0.88 (Baruchin and Ladizinsky 1983).

Chromosome segregation at AI was examined in 160 cells. In 134 of them 7-8 segregation was observed and 7-7 in the rest, indicating that about 42% of the meiotic products will contain eight chromosomes. In the 7-7 segregation the extra chromosome remained in the equatorial plate (Fig. 2) and later formed a micronucleus. Of the 52 tetrads that were scored 44 were normal and 8 contained micronuclei. The average pollen fertility in the

trisomic plants was 51% but it ranged from 9 to 75% among plants (Table 2).

The transmission rate of the extra chromosome in the seeds was 29% (Table 1). It was checked further in second generation, this time the chromosome number was counted in 204 seedlings and only 56 had $2n=15$ which indicates transmission rate of 22.5%.

Trisomics of *L.culinaris*, no.2 X *L.orientalis*, no.42 cross

The F_1 hybrid of this cross combination was heterozygous for a reciprocal translocation involving two submetacentric chromosomes. Chromosome number was checked in 312 F_2 plants and two had $2n=15$. As expected the extra chromosome was submeracentric in both trisomic plants. These plants were very weak and died before seed setting.

Trisomics of *L.culinaris*, no.2 X *L.orientalis*, no.133 cross

Hybrids of this combination were difficult to produce because the hybrid embryos collapse 7-14 days after pollination. Only rarely viable seeds were produced, but a large number of hybrids could be obtained by culturing the immature embryos in artificial medium (Cohen et al.1984). Chromosome pairing in the mature hybrids was extremely irregular and multivalents and univalents were observed at metaphase I. The highest number of multivalents per cell indicated that the parental lines differed from each other by at least four chromosomal rearrangements. Consequently, the fertility was very low. Chromosome number was checked in 102 F_2 plants and $2n=15$ was found in 11 of them. Chromosome association at meiosis in these trisomic plants was

irregular and indicated that they were heterozygous to 1-3 translocations. Pollen fertility of these plants was very low and most of them did not set even a single seed.

Trisomics of *L.culinaris*, no.2 X *L.ervoides*, no.32 cross

Hybrids of this cross combination can be obtained only by the aid of embryo culture (Cohen et al.1984, Ladizinsky et al. 1985). The F_1 hybrid was heterozygous to a single reciprocal translocation involving submetacentric and acrocentric chromosomes. Chromosome number was checked in 207 F_2 seedlings and two had $2n=15$. The extra chromosome was submetacentric in one and acrocentric in the second (Fig. 2). Both died at seedling stage.

Discussion

The inviability of autotriploid embryos makes the production of primary trisomics in lentil extremely difficult. Trisomic individuals, however, may be found among progeny of translocation heterozygotes. Chromosomal rearrangements are not common in the cultivated lentil but can be induced by irradiation. On the other hand they are quite common in the wild progenitor *L.orientalis* (Ladizinsky et al 1984). Thus, for systematic search for tertiary trisomics it may be useful to examine derivatives of hybrids between accessions of *L.orientalis* or between them and the cultivated lentil

Characteristically, all the trisomic plants obtained in this study had retarded growth compared with disomic plants. Many of

It appears that if trisomic lines are to be successfully used for genetic analysis in lentil a better way of keeping them alive and making them more productive must be developed. Tertiary trisomic plants were obtained from three different hybrids, each of them heterozygous for a different reciprocal translocation. Gametes with $n+1$ chromosome number in such hybrids may originate from inappropriate segregation of the chromosomes involved in the reciprocal translocation. The proportions of the tertiary trisomic plants among the derivatives of the F_1 hybrids heterozygous to a single reciprocal translocation was 0.6%-1.6%. A much higher proportion of trisomic derivatives was found in L.culinaris, no.2 X L.orientalis no.133 family. These plants were also heterozygous to one or two translocations, highly sterile and useless as genetic stocks. It demonstrates that a search for trisomic plants should be confined to derivatives of F_1 hybrids which are heterozygous to a single translocation.

The meiotic behavior and the transmission rate of the extra chromosome could be studied in trisomic plants which derived from L.culinaris, no.13 X L.orientalis, no.24 cross. The extra chromosome was involved in almost all the possible meiotic configurations, except heteromorphic bivalent involving the translocated and the acrocentric chromosomes, and heteromorphic trivalent involving two submetacentric and one acrocentric chromosomes. Since chiasmata in the native and the translocated arms of the extra chromosome were in nearly similar proportions it is likely that it also did not form any bivalent with one of

the native submetacentric chromosomes. The kind and the proportions of configurations in which the extra chromosome was involved can be used to estimate the likelihood of primary trisomic production by the tertiary trisomic plants. Obviously, no $n+1$ gamete which includes the two homologous native submetacentric chromosomes could be formed from cells with pentavalent or V shaped trivalent configurations. These gametes may be formed, however, as a result of segregation of homomorphic or heteromorphic chain trivalents. Of the 261 MI cell examined 17 had homomorphic chain trivalents. The translocated chromosome iwa always terminal in these trivalents and at AI the two native submetacentric chromosomes, and a native and the translocated chromosome migrated to the same pole in about equal proportions. Accordingly, it is expected that about 3% of the gametes of the tertiary trisomic plants would contain two homologues of submetacentric chromosomes and therefore would be capable of producing primary trisomics. These primary trisomics can be detected only according to their pairing pattern at MI. So far we have examined chromosome association at MI of more than 60 trisomic plants but all appeared to be tertiary trisomics. Only one of the examined MI cells contained a heteromorphic chain trivalent and thus about 0.2% of the gametes are expected to possess two homologues of the acrocentric chromosome and could give rise to primary trisomics of the acrocentric chromosome.

Chromosome segregation at AI indicated that $n=7$ and $n=8$ gametes were produced at about equal proportions. Since, on the

average, pollen fertility of the trisomic plants was 50% we assume that the transmission of the extra chromosome was basically via the female gametes. The transmission rate in the seeds showed some variation among plants, 0.24-0.35, and somewhat lower in the next growing season, probably due to some environmental effects. Obviously, only reciprocal crosses of the tertiary trisomics with $2n=14$ plants would indicate the exact transmission rate of the extra chromosome through the male and female gametes.

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Table 1. Transmission rate of the extra chromosome in C13/024 tertiary trisomic.

Trisomic parental plants	2	3	4	total
No. seeds planted	67	70	30	167
No. seeds germinated	62	69	21	152
No. $2n=14$ seedlings	48	46	13	107
No. $2n=15$ seedlings	15	21	8	44
Transmission rate	0.24	0.30	0.35	0.29

Table 2. Chromosome association at MI and pollen fertility in trisomic plants.

Plant No.	No. cells	Chromosome associations				% Pollen fertility
		7II+I	6II+III	5II+V	5II+IV+I	
2-42	24	8	16			45
2-44	22	3	16	3		43
2-46	11	1	2	8		65
2-58	18	5	9	4		60
2-65	11	3	6	1	1	9
3-08	20	2	9	9		54
3-09	15	3	8	4		54
3-10	5	1	2	2		37
3-16	5		4	1		56
3-19	7	1	5	1		67
3-20	40	4	19	17		62
3-37	12	4	3	5		43
3-43	14	4	5	5		48
4-06	20	6	8	6		75
4-08	11	2	6	2	1	61
4-13	15	4	11			57
4-16	11	2	5	4		38

Explanation of figures

Fig. 1: A slit in the ventral side of the pod in C13/O24 trisomic.

Fig. 2: Mitotic and meiotic chromosomes in lentil trisomics.

A. $2n=15$ in C13/O24, the extra chromosome is submetacentric.

B. $2n=15$ in E32/C7, the extra chromosome acrocentric.

C. Homomorphic trivalent in a $2n=15$ plant.

D. Pentavalent in a $2n=15$ plant.

E. Heteromorphic trivalent in a $2n=15$ plant.

F. The extrachromosome in the equatorial plate at telophase I.

Fig. 1 (Lod. sp.)

**DUPLICATION OF ALDOLASE AND ESTERASE LOCI IN Cicer
(Cicereae Alef.)**

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Running title: Duplication of isozyme loci in Cicer

The genetic control of fructose bisphosphate aldolase (ALDO, EC 4.1.2.13), and esterase (EST, EC 3.1.1.2.) isozymes in Cicer was studied by starch gel electrophoresis. Fixed heterozygote enzyme phenotype in homozygotes and asymmetrical banding intensities in heterozygotes were observed for both Aldo-1, Aldo-2 and Est-4, and Est-5 in all the self-pollinated Cicer species examined. Subcellular localization studies demonstrated that the products of duplicated aldolase loci are present in the plastids, whereas duplicated esterase isozymes were found in the cytosolic compartment. Selfing and crossing experiments revealed that there are two nuclear genes encoding plastid specific ALDO isozymes, (Aldo-1 and Aldo-2), but the location of these genes relative to other known markers is not known. Similarly, EST-4 and EST-5 isozymes are specified by two nuclear genes (Est-4 and Est-5). Linkage relationships between the duplicated aldolase and esterase isozymes are under investigation but no linkage was found between any of the duplicated genes and the other isozyme loci, Aco, Adh, Gpt, Gpi, Pgd, Pgm, Sp, and Gal, examined in this study. Three alleles were detected for each aldolase locus in the electrophoretic survey of related species, whereas two alleles were found for duplicated esterase locus (Est-5). Present evidence suggests that these duplications are unique and probably occurred only in this monophyletic tribe, Cicereae, since no duplication was reported in the related genera. No evidence for mutations silencing any of the duplicated copies was detected in the genus; therefore, this duplication cannot be considered ancient. Although the

mechanism for duplications is not known, evidence for translocations in Cicer, and the existence of a similar linkage between ALDO and EST isozymes in related genera indicate that both duplications may have arisen simultaneously via duplication of a chromosomal segment carrying the ancestral state of the genes.

Gene duplications have been reported in a number of plant (Cai and Chinnappa 1989; Ellstrand et al. 1983; Goldring et al. 1985; Gottlieb 1977; 1987; Gottlieb and Weeden 1979; Odrzykoski and Gottlieb 1984; Pichersky and Gottlieb 1983; Roose and Gottlieb 1980; Soltis et al. 1987), and animal (Allendorf et al. 1975; Avise and Kitto 1973) species. Polyploid plants normally have duplicated isozymes as a consequence of genome duplication (Hart 1975; Lumaret 1986; Martinez-Zapater and Oliver, 1985). However, duplications of nuclear genes encoding the enzymes functioning in the glycolytic pathway have also been reported in a number of diploid plant species (Gottlieb 1977; Odrzykoski and Gottlieb 1984; Pichersky and Gottlieb 1983; Soltis et al. 1987). Maize, although usually considered as a diploid plant, was found to have duplicated (McMillin and Scandalios, 1980; 1981; Wendel et al., 1986; Yang et al. 1977), and even triplicated (Wendel et al. 1989) isozymes that have been observed for polyploid plant species. A great number of chromosomal segments are redundant in maize as revealed by RFLP analysis (Helentjaris et al. 1988). All this evidence led some authors to propose that allopolyploidy may have been involved in the evolution of maize (Helentjaris et al. 1988; Wendel et al. 1989).

Gene duplication is important in evolution because it appears to be one of the main mechanisms for the acquisition of new genes and consequently new metabolic capabilities (Gottlieb 1982; Markert et al. 1975). Accordingly, when a gene is duplicated, one of the two resultant genes may become non-functional or acquire a

new function (Allendorf 1978; Allendorf et al. 1984; Takahata 1982). A duplicated locus may be fixed in diploid populations by mutation pressure and random drift (Kimura and King 1979). Gene silencing may especially occur after polyploidisation since many enzyme loci are the parts of a redundant gene pool in polyploid species (Ferris and Whitt 1977; Wilson et al. 1983; Wolko and Weeden 1989). However, currently very little is known about how duplicated genes may diverge in regulation or function.

More solid evidence for gene duplication requires demonstration of similar amino acid sequences from both duplicates or cytological demonstration of duplicated chromosome material with their isozyme coding genes (Gottlieb 1982). However, electrophoretic studies can also reveal the multilocus nature of many enzyme systems, providing support for the gene duplication hypothesis (Ellstrand et al. 1983).

Aldolase (EC 4.1.2.13.), functioning in the glycolytic pathway catalyzes the reversible conversion of fructose diphosphate to glyceraldehyde 3 phosphate. Similar to other glycolytic pathway enzymes, two aldolase isozymes localized in plastidic and cytosolic compartments were found in higher plants (Anderson and Levin 1970; Bukowiecki and Anderson 1974). Fluorescent esterase (EST, EC 3.1.1.2.) is associated with the biosynthesis of acetate (Doong and Kiang 1988). The presence of several esterase isozymes are known in the plants (Clegg and Allard 1973; Doong and Kiang 1988; Weeden and Marx 1987). In this report, we present evidence that duplication of the gene for plastid specific aldolase isozymes and one of the

esterase isozymes have occurred during the evolution of Cicer (Cicereae Alef.), consisting of diploid ($2n=16$) and self-pollinated annual and perennial species.

MATERIALS AND METHODS

Plant Material: True breeding diploid chickpea lines maintained by natural self-pollination were used to recognize the nature of the fixed heterozygote phenotype. An alternative allele for one of the duplicated loci (Aldo-1) was found in a wild species of chickpea, Cicer reticulatum. Segregation of aldolase isozymes was examined in the progeny of a cross (Family A) between C. arietinum (PI360177) and C. reticulatum (PI489777). Polymorphism for the duplicated esterase locus was found in two C. arietinum accessions and segregation of this locus was examined in the progeny of a cross (Family B) between two accessions, ILC 194 and FLIP 86-83. Additionally, the taxonomic distribution of duplications was determined by an electrophoretic survey of all remaining annual species (C. reticulatum Lad. [PI489777, PI489778, PI510655], C. echinospermum Dav. [PI489776], C. bijugum Rech. [PI458550, PI458551, PI458552], C. pinnatifidum J. [PI458555, PI458556, PI510663], C. judaicum Boiss. [PI458558, PI458559, PI504291], C. yamashitae Kitam. [PI504550, PI510664], C. chorassanicum (Bge) M. Pop. [PI458553], C. cuneatum Rich. [PI458554]) and one perennial species (C. anatolicum Alef. [PI383626]) of Cicer obtained from United States Department of Agriculture Western Regional Plant Introduction Station, Pullman, WA.

Enzyme Assays and Electrophoresis: Enzyme extracts were obtained by grinding two or three leaflets of young seedlings in a 100 μ l potassium phosphate, pH 7, extraction buffer according to Soltis et al. (1983). For alcohol dehydrogenase (ADH, EC 1.1.1.1), extractions from roots soaked 18 hours in water were used. A pH 6.1 histidine gel and electrode buffer (Cardy et al. 1980) was used to resolve alcohol dehydrogenase, aldolase (ALDO, EC 4.1.2.13), 6-phosphogluconate dehydrogenase (PGD, EC 1.1.1.44), and phosphoglucomutase (PGM, EC 2.7.5.1). A tris-citrate/lithium borate pH 8.1, gel system (Selander et al. 1971) was used to resolve aspartate aminotransferase (AAT, EC 2.6.1.1), aconitase (ACO, EC 4.2.1.3), glucose-1-phosphate transferase (GPT, EC 3.1.3.10), glucose-6-phosphate isomerase (GPI, EC 5.3.1.9) and seed protein (SP). A citrate/N-3-aminopropyl-morpholine gel system, pH 6.1, (Clayton and Tretiak 1972) was used to resolve fluorescent esterase (EST, EC 3.1.1.2), and β -D-galactosidase (GAL, EC 3.2.1). Electrophoresis was carried out using a 10% starch gel for all the gel systems. The gel was run for four hours at a constant voltage of 310 for the assays conducted on the histidine gel system. For the other assays, the gel was run at 45 mA for four hours in a refrigerator cabinet. The SP was assayed with a naphtol blue black solution (5:5:1, methanol, water and acetic acid). The remaining assays were identical or slight modifications of those described by Soltis et al. (1983).

Subcellular Location of Isozymes: Subcellular location of the isozymes was determined by comparing the electrophoretic

patterns of pollen leachate, leaf extract, chloroplast, and mitochondrial preparations as described by Muehlbauer et al. (1989) and Weeden and Gottlieb (1980).

Isozyme nomenclature: The locus specifying the most anodally migrating isozyme was designated as 1, the next 2 and so on. Similarly, the alleles at each locus were named according to the relative mobilities of their products. The most anodal allozyme at a given locus was assigned with "a", whereas the subsequent letters were used to indicate the slower migrating allozymes. The letter, p, c, or m, was used after the name of the locus to indicate the subcellular location of isozymes as plastid, cytosol and mitochondria, respectively.

Segregation and Linkage Analysis: Parental lines maintained by self-pollination were selfed to demonstrate the homozygous nature of the plants. Crosses between the plants differing for the alleles in one of the duplicated loci for aldolase (1aa2cc x 1cc2cc) and esterase (1aa2aa x 1aa2bb) were made and F₂ progenies were tested for deviation from expected Mendelian segregation ratios for a single locus. The LINKAGE-1 computer, program made available by Suiter et al. (1983), was used for linkage analyses between Aldo-1 and nine other isozyme loci, Aco-c, Aco-m, Pgd-c, Pgi-p, Pgm-p, Gpt-p, Gpt-c, Sp, and Adh-1, segregating in Family A and between Est-5 and Gal-1 segregating in Family B.

RESULTS

Fixed Heterozygosity: The presence of true breeding individuals with multiple-banded electrophoretic patterns suggested

the presence of duplicate genes coding for aldolase. A 5-banded aldolase pattern was observed in C. arietinum, whereas only a very darkly stained region was found in C. reticulatum (PI489777). Both phenotypes did not segregate upon selfing, indicating that this is a fixed heterozygote phenotype (existence of multiple banded enzyme patterns of homozygous individuals that can normally be observed for heterozygous individuals) encoded by two distinct genes. However, segregation was obtained in crosses between the two species sharing one of the alleles for duplicated locus but differing for the other (Figure 1). The enzyme genotypes of C. arietinum, and C. reticulatum were designated as 1aa2cc and 1cc2cc, respectively (Figure 3a,3c). In F₁ hybrids, a 5-banded pattern for aldolase was observed, indicating the tetrameric nature of the enzyme. The F₁ genotype was designated as 1ac2cc (Figure 3b). One- and three-banded phenotypes for EST-4,5 region were found in two C. arietinum accessions used as parents in the crosses (ILC-194 x FLIP 86-85) (Figure 2). The genotypes of the parents were designated as 4aa5aa for 1-banded and 4aa5bb for three banded phenotypes (Figure 3d,3f). Selfing of the individuals with three banded pattern did not show segregation. The crosses between these two genotypes yielded a 3-banded phenotype (4aa5ab) with two darkly stained bands and a fainter band at the third position (Figure 3e).

Intergenic Interaction Producing Hybrid Isozymes: Intergenic heterotetramers produced by the subunit association of the alleles at the duplicated Aldo loci were observed in all the species examined. Intergenic bands produced by random association of the

polypeptides from duplicated genes indicate the structural similarity of the polypeptides from both genes. Since duplicated isozymes are expressed in the same subcellular compartment (plastid), intergenic interaction can easily occur between the isozymes, producing multiple bands. No distinguishable intergenic band was observed in C. reticulatum since the alleles at both loci have the same electrophoretic mobility for duplicated isozymes. No intergenic interaction was observed between cytosolic aldolase isozyme (Aldo-3) and the duplicated plastid specific isozymes (Aldo-1 and Aldo-2).

Similarly, a three-banded enzyme phenotype was produced by subunit association of the genes encoding EST-4 and EST-5 isozymes. One-banded phenotype observed in one of the parents was probably due to the fact that the products of both loci overlap on the gel. We found all esterase isozymes exclusively localized in the cytosol. Existence of both isozymes in the same subcellular compartment allows products of the duplicated loci to combine and produce a fixed heterozygote phenotype. No intergenic interaction between any other esterase loci products was observed. This suggests that these loci have diverged.

Gene Dosage Effect in Heterozygotes: Asymmetrical banding intensities were observed for heterozygous individuals for both duplicated esterase and aldolase loci (Figure 1 and 2). In duplicated esterase locus a allele encodes a fast and b allele encodes a slow allozyme. Accordingly, the fast band will be more intense in an individual with 4aa5ab genotype because of the dosage

effect of a. Similar observation was made for heterozygous individuals (1ac2cc) at the duplicated aldolase locus. Due to dosage effect of c, three relatively darker staining bands with a relatively lighter staining portion was observed in the heterozygote. This situation was proposed as evidence for gene duplication (Cai and Chinnappa 1989; Martinez-Zapater and Oliver 1985; Oliver et al. 1983; Weeden et al. 1989).

Taxonomic Distribution of Gene Duplications: The alleles at duplicated aldolase loci are illustrated graphically in Figure 4. No variation was found within each species except C. reticulatum, which showed one- (PI489777) and 5-banded (PI489778 and PI510655) phenotypes for the different alleles of the duplicated genes. Different alleles were fixed for most of the species examined. Three alleles were detected for both Aldo-1 (1a,1b,1c) and Aldo-2 (2a, 2b, 2c) in a survey of all annual and one perennial (C. anatolicum) species of Cicer. Species in the first two crossability groups, except C. reticulatum (PI489777) shared the same alleles for both Aldo-1 and Aldo-2. Similarly, C. cuneatum and C. reticulatum had the same allele for Aldo-2 but they differed from the remaining species for the Aldo-1 allele. Two other wild species, C. yamashitae, and C. anatolicum differed from the other species by having the same allele for Aldo-2.

Two alleles were found for the duplicated Est-5 in the three species of the genus, C. arietinum (5a and 5b), C. reticulatum (5a), and C. echinospermum (5a). Complex banding patterns for esterase isozymes in the remaining species prevented further

evaluation of this duplication.

Genetic Control of ALDO and EST Phenotypes in Cicer: Self pollination of individuals within the parental lines yielded progeny having the same aldolase phenotypes as the parents, indicating the homozygosity of the parents. However, the cross between them produced F_1 progeny which consisted of both parental and heterotetrameric enzymes. This is due to the fact that both parents have the same allele for the less anodal plastid isozyme (ALDO-2); however they differ by having different alleles for the more anodal (ALDO-1) plastid specific isozymes. Three progeny classes occurred in the F_2 , 5- and 1-banded parental phenotypes and 5-banded F_1 phenotype. The number of individuals in the progeny classes approximated a 1:2:1 ratio expected for codominant alleles segregating at a single locus (Table 1).

Six zones of activity for esterase were observed. The two most anodal isozymes are monomeric and under the control of two linked loci (Kazan and Kusmenoglu unpublished). The genetic control of third and sixth most anodal isozymes are not known. The present study revealed that zone 4 and zone 5 isozymes are controlled by a duplicated locus. Variation was found for only zone 5 (EST-5) isozymes in the crossable parents. Crosses between the parental genotypes differing by the alleles for zone 5 isozymes yielded both parental and heterozygote phenotype in F_2 and segregation fit the expected 1:2:1 ratio for a single locus (Table 1).

Linkage Tests: Testing the linkage between duplicated aldolase loci was not possible since only one locus showed

variation in the parents. Testing the same linkage in some other Cicer species carrying different alleles for both Aldo-1 and Aldo-2 was not possible due to the crossability barriers.

Tests of linkage between Aldo-1 and nine isozyme loci are presented in Table 2. Aldo-1 segregated independently from the other loci tested. All other isozyme loci exhibited codominant expression of allozymes and gave a 1:2:1 segregation ratios in the F_2 .

Testing the linkage between duplicated esterase loci was also not possible due to lack of polymorphism for both duplicated copies in the parental genotypes. Although the linkage between Gal-1 and Est-5 was tested, no linkage was found (Table 2).

DISCUSSION

Evidence was presented for duplication of two enzyme loci in Cicer. The presence of a fixed heterozygote enzyme phenotype, intergenic interaction producing heteromeric isozymes, and the presence of the duplicated isozymes in the same subcellular compartments all indicate the duplicated states of the genes. Additionally, we have evidence to suggest a possible segment duplication giving rise to the duplicated isozymes in this genus. Although relatively rare, duplication of a segment of a chromosome carrying genes encoding cytosolic and mitochondrial malate dehydrogenase isozymes has been reported in maize (McMillin and Scandalios 1980). Cytosolic Mdh-1 and Mdh-2 were located on different maize chromosomes and both Mdh-1 and Mdh-2 were found to be linked to two aminopeptidase loci (Amp-1, and Amp-2). This

suggested that the region including Mdh-c and Amp may have been duplicated (McMillin and Scandalios 1980; 1981). Although we do not have a direct evidence suggesting a linkage between duplicated aldolase and esterase loci in Cicer, a similar linkage between these two loci was reported in Pisum (Weeden and Marx 1987). The same linkage situation may exist in Cicer since the presence of several conserved linkage groups consisting of homologous genes among the members of Viceae has been demonstrated (Muehlbauer et al. 1989; Weeden et al. 1988; Kazan et al. unpublished). We are currently attempting to demonstrate a similar linkage group in Cicer. The presence of a linkage between Aldo and Skdh is also known in Pisum (Weeden and Marx 1987). In contrast to Pisum and Lens, for which only one SKDH isozyme was reported, two SKDH isozymes were resolved in Cicer. The duplication of Aldo and Est loci and putative duplication of a Skdh locus suggests the possible duplication of a chromosome segment carrying the ancestral state of the duplicated genes. No polymorphism was found within each crossability group of Cicer for Skdh to test the possibility of a linkage among these three loci (Kazan unpublished).

Gottlieb (1982) proposed a mechanism by which gene duplication may occur. Accordingly, most of the duplications with unlinked copies may have arisen from the plants which were heterozygous for overlapping reciprocal translocations. Duplicate genes were found unlinked for most of the instances suggesting that they do not usually originate by unequal crossing over. Gene duplications fitting proposed mode of origin were observed for a

number of isozyme gene duplications such as phosphoglucose isomerase, and triose phosphate isomerase in *Clarkia* (Gottlieb 1977; Pichersky and Gottlieb 1983), phosphoglucomutase in *Capsicum annuum* (Tanksley 1984), and 6-phosphogluconate dehydrogenase in tomato (Tanksley and Kuehn 1985). As apparent from the model, chromosomal rearrangements are precursors for the gene and segment duplications. Several chromosomal rearrangements have also been identified in *Cicer* (Ladizinsky and Adler 1976). For example, a translocation, a reciprocal translocation, and a paracentric inversion were found in the hybrids made among the three compatible species, *C. arietinum*, *C. reticulatum*, and *C. echinospermum*. Although cytogenetic evidence for translocations is not currently available for other species, other data suggest the possible role of chromosomal rearrangements in the evolution of the genus. Possibly such rearrangements gave rise to the duplicated segment after *Cicer* diverged from the related taxa.

Allendorf (1978) has discussed the possible fate of duplicated genes. The main reason for conservation of duplicated loci would be selective advantage of the individuals with the gene duplications. Since the number of isozymes is increased in fixed heterozygote individuals, larger amounts of enzyme with slightly different biochemical properties may be produced. The difference between the individuals with and without gene duplication might be very distinct in nature especially under adverse conditions. This might be true for the aldolase gene duplication although we do not know the biochemical properties of additional isozymes developed

from gene duplication. However, the esterase duplication will probably be silenced because duplicated isozymes are already a part of a redundant enzyme system.

Gene duplications have also been used extensively in plant systematics to clarify the evolutionary relationships among taxa (Gottlieb 1977; Gottlieb and Weeden 1979; Odrzykoski and Gottlieb 1984; Soltis et al. 1987) due to the fact that they are very rare events and probably occur only once in the evolution of a genus. Similarly, loss of duplication in some species within a genus suggests the phylogenetic similarity among them. The gene encoding the cytosolic GPI, Gpi-c, was found duplicated within the Leguminosae (Weeden et al. 1989). The distribution of this gene duplication among the members of the family led to the conclusion that the duplication had occurred in the lineage leading to Phaseolus after its divergence from that leading to Pisum and Lens. Similarly, restriction of present duplications to Cicer, a monophyletic genera in the Cicereae, suggests that the duplication probably occurred after the divergence of the tribe from other related taxa.

The distribution of a gene duplication among the related taxa may also reveal some information regarding the age of a duplication. For example, the present duplications involving ALDO and EST cannot be considered ancient, since no duplication of these two loci has been reported in related genera. This suggests that the duplicated enzyme loci are still at the early stages of differentiation, since they produce polypeptides that are

structurally and metabolically similar, allowing the formation of heteropolymers.

Genetic and biochemical evidence suggest that the duplication of plastid specific aldolase and an esterase locus occurred by a process of gene and possibly a segment duplication. However, biochemical evidence of structural and sequence similarity for the genes or their products is not yet available. Currently, ancestral state of the genes is also not known.

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Table 1: Segregation of duplicated isozyme phenotypes in F₂ progenies of an interspecific hybrid (Family A: PI360177 x PI489777), and an intraspecific hybrid (Family B: ILC-194 x FLIP-86-83), and single locus goodness of fit to a 1:2:1 expected genetic ratio.

No. of plants with designated phenotype						

Family	Locus	Fast	Heterozygotes	Slow	X ²	P

A	Aldo-1 (p)	16	28	15	0.19	0.91
B	Est-5	38	89	33	2.33	0.31

Table 2: Joint segregation between Aldo-1 and nine other isozyme loci in Family A and between Est-5 and Gal-1 in Family B and tests for linkage.

		No. of F ₂ Plants with Designated Phenotypes ^a											
Family	Locus	FF	FH	FS	HF	HH	HS	SF	SH	SS	X ²	r ^b	P
A	Aco-c	5	8	2	8	13	8	3	6	5	2.05	42	0.73
	Aco-m	4	7	1	7	16	9	5	4	5	4.28	44	0.37
	Pgd-c	0	12	6	11	8	7	5	7	2	11.50	39	0.02
	Pgi-c	5	11	3	7	13	9	4	3	3	2.94	48	0.57
	Pgm-p	3	5	2	6	5	11	7	7	2	4.63	41	0.33
	Gpt-p	5	6	5	8	13	7	3	8	3	1.19	49	0.88
	Gpt-c	3	11	1	11	9	7	2	7	7	10.79	41	0.03
	Adh-1(c)	5	7	5	9	9	7	2	11	3	4.82	49	0.31
	Sp	4	11	2	10	13	10	2	3	3	3.89	42	0.42
B	Gal-1	10	20	8	20	46	23	13	15	5	4.06	45	0.03

^a F=homozygous fast

S=homozygous slow

H=heterozygous

^b recombination fraction

Figure 1: Segregation of isozyme phenotypes for Aldo-1 in an interspecific cross (see text for details; F=fast, S=slow, H=heterozygote enzyme phenotype).

Figure 2: Segregation of isozyme phenotypes for Est-5 in an intraspecific cross (see text for details; F=fast, S=slow, H=heterozygote enzyme phenotype).

Figure 3: Diagrammatic representations of the electrophoretic phenotypes for aldolase; a=1aa2cc (C. arietinum, PI360177), c=1cc2cc (C. reticulatum, PI489777) parental and b=1ac2cc heterozygous, and for esterase isozymes; d=1aa2aa (C. arietinum, ILC-194) f=1aa2bb (C. arietinum, FLIP 86-83) parental, and e=1aa2ab heterozygous. Only duplicated isozymes are shown.

Figure 4: Diagrammatic representation of aldolase isozymes in Cicer species. a) 1aa2bb (C. chrossanicum), b) 1aa2cc (C. arietinum, C. reticulatum [PI489778, PI510655], C. echinospermum, C. bijugum, C. pinnatifidum, C. judaicum), c) 1aa2aa (C. yamashitae, C. anatolicum), d) 1bb2cc (C. cuneatum), e) 1cc2cc (C. reticulatum [PI489777]).

F. DESCRIPTION OF COLLABORATION

The initial research objectives were identified and partitioned to take advantage of the areas of expertise of each investigator. Thus, Dr. Ladizinsky focused on developing trisomic lines, Dr. Muehlbauer on the generation of appropriate lentil and chickpea progeny for linkage analysis, and Dr. Weeden on the analysis of segregating RFLP markers. However, communication among the PIs allowed each PI to contribute significantly to the major goal of the project--the formation of a linkage map. The interspecific cross in *Lens* was originally developed in Dr. Ladizinsky's program and was investigated by Dr. D. Zamir before being identified by Dr. Weeden as the most promising cross for his own work. The set of cDNA clones made by Dr. M. Havey in Dr. Muehlbauer's program proved to be the most useful source of polymorphic DNA markers for both the *Lens* and the *Cicer* mapping studies.

After approximately a year and a half into the project all three PIs met in Pullman, WA to discuss the progress achieved in the several lines of investigation and combine expertise to perform techniques such as *in situ* hybridizations which no one PI had the background to do alone. This month of interaction proved to be both a stimulating and productive experience for each of the PIs. Dr. Muehlbauer showed that the F2 progeny from the *Cicer arietinum* x *C. reticulatum* cross was segregating for many allozyme polymorphisms and, hence, would make a suitable progeny for the RFLP analysis. We had not been able to identify much genetic diversity at the molecular level within *C. arietinum* and were seriously considering dropping the objective of developing a linkage map in *Cicer*. Also during this time Dr. Ladinsky and Dr Weeden used

biotin labeled probes for *in situ* hybridization experiments. The location of the nucleolar organizer regions could be clearly seen using this technique.

Dr. Weeden spent another eight months in Pullman as part of his sabbatical leave. During this time part of the RFLP mapping in *Lens* and all of that in *Cicer* was completed. The PIs continue to collaborate on several studies that were initiated during the funding period.

G. EVALUATION OF THE RESEARCH ACHIEVEMENTS

Most of the original goals of the proposal were attained. An extensive linkage map for lentil was developed as was a partial one for chickpea. When these results, and recent data from other mapping studies on lentil and chickpea, are compared to the linkage map already established for the garden pea considerable synteny can be observed, especially between lentil and garden pea. These latter two species represent two divergent line within the Viceae. Linkage groups shared by these two taxa also would be expected to be present in intermediate taxa such a *Vicia faba* and *Lathyrus sativus*. The evidence suggests that certain of these linkage groups also are conserved in chickpea.

This technique of interpolation and extrapolation provides a method for generating partial linkage maps in crops such a faba bean and sweet pea. The linkage groups Est---Skdh---Cab, CMH3---Lap-1, Aat-p---Lb, and Pep---Fk---Pgd-p---Pgm-c are almost certainly present in each of the latter crops. The technique also permits analysis of homology between similar characters in different taxa. The gene G_s, which confers a red (anthocyanin) pigmentation to portions of the epicotyl in lentil, is probably homologous to D in garden pea, which also produces red pigmentation on the stem but is better known for the production of anthocyanin deposits in the leaf axils. The finding that both genes are linked to the leghemoglobin gene cluster in the respective species greatly strengthens the case for their homology.

We did not examine the genetic diversity in *Cicer* except to note that the diversity within the cultivated species was low as measured both by allozyme and DNA restriction fragment length polymorphism. The generation of trisomic stocks was not as easy as we had hoped, and only

one tertiary trisomic was vigorous enough to be maintained through several generations.

Thus, the major benefit to agriculture of our result will be the establishment of a generalized linkage map for the Viceae and more detailed maps for lentil and chickpea. We have recombinant inbred F_6 lines for at least 50 of the F_2 plants in the *L. culinaris* x *L. ervoides* progeny. These lines should be particularly useful for further mapping of DNA probes and several quantitative traits.

H. LIST OF PUBLICATIONS AND PAPERS IN PREPARATION

Kazan, K., I. Kusmenoglu, F. J. Muehlbauer and N. F. Weeden
(submitted to J. Heredity). Duplication of aldolase and
esterase isozyme loci in *Cicer* (Cicereae Alef.).

Kazan, K., F. J. Muehlbauer, N. F. Weeden and G. D. Ladizinsky
(in review) Inheritance and linkage relationships of
morphological and isozyme loci in chickpea (*Cicer
arietinum* L.).

Ladizinsky, G., N. F. Weeden, and F. J. Muehlbauer (submitted to
Euphytica) Tertiary trisomics in lentil.

Weeden, N. F. and J. F. Wendel. 1989. Genetics of plant isozymes.
In: Isozymes in Plant Biology (D. Soltis and P. Soltis, eds.).
Dioscorides Press, Portland. pp. 46-72.

Weeden, N. F., F. J. Muehlbauer, and G. Ladizinsky. (in review)
Development of general linkage maps for *Lens* and *Cicer*
from interspecific crosses and comparison of these with
the linkage map for *Pisum*.